=> d his full

(FILE 'HOME' ENTERED AT 08:12:06 ON 06 AUG 2006)

INDEX 'ADISCTI, ADISINSIGHT, ADISNEWS, AGRICOLA, ANABSTR, ANTE, AQUALINE, AQUASCI, BIOENG, BIOSIS, BIOTECHABS, BIOTECHDS, BIOTECHNO, CABA, CAPLUS, CEABA-VTB, CIN, CONFSCI, CROPB, CROPU, DDFB, DDFU, DGENE, DISSABS, DRUGB, DRUGMONOG2, DRUGU, EMBAL, EMBASE, ...' ENTERED AT 08:12:22 ON 06 AUG 2006

SEA GLUCOSAMIN?(S)(SYNTHE? OR PRODUC? OR PROCE? OR MANUFAC? OR

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21 FILE ADISCTI
25 FILE ADISINSIGHT
30 FILE ADISNEWS
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2251 FILE MEDLINE
39 FILE NTIS
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45 FILE OCEAN
1594 FILE PASCAL
9 FILE PHAR
4 FILE PHARMAML
88 FILE PHIN
986 FILE PROMT
12 FILE PROUSDDR
7 FILE RDISCLOSURE
1788 FILE SCISEARCH
11 FILE SYNTHLINE
1158 FILE TOXCENTER
4724 FILE USPATFULL
483 FILE USPAT2
2 FILE VETB

41 FILE VETU
22 FILE WATER
871 FILE WPIDS
7 FILE WPIFV
871 FILE WPINDEX
48 FILE IPA
10 FILE NAPRALERT
506 FILE NLDB

L1 QUE GLUCOSAMIN?(S)(SYNTHE? OR PRODUC? OR PROCE? OR MANUFAC? OR BIOSYNTH?)

D RANK

FILE 'USPATFULL, CAPLUS, BIOSIS, MEDLINE, EMBASE, BIOTECHNO, SCISEARCH, ESBIOBASE, PASCAL, LIFESCI' ENTERED AT 08:20:19 ON 06 AUG 2006

L2 25082 SEA GLUCOSAMIN?(S)(SYNTHE? OR PRODUC? OR PROCE? OR MANUFAC? OR BIOSYNTH?)
L3 225 SEA L2(S)(ACETYLTRANSFERAS?)
L4 134 SEA L3(S) PHOSPHAT?
L5 95 DUP REM L4 (39 DUPLICATES REMOVED)
L6 64 SEA L5(S)(SYNTHETAS? OR SYNTHAS?)
D TI L6 1-64
D L6 IBIB ABS 6 17 24 51 54 58
D IBIB ABS L6 3

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 NEWS  3  FEB 27   New STN AnaVist pricing effective March 1, 2006
 NEWS  4  APR 04   STN AnaVist $500 visualization usage credit offered
 NEWS  5  MAY 10   CA/CAplus enhanced with 1900-1906 U.S. patent records
 NEWS  6  MAY 11   KOREAPAT updates resume
 NEWS  7  MAY 19   Derwent World Patents Index to be reloaded and enhanced
 NEWS  8  MAY 30   IPC 8 Rolled-up Core codes added to CA/CAplus and
                   USPATFULL/USPAT2
 NEWS  9  MAY 30   The F-Term thesaurus is now available in CA/CAplus
 NEWS 10  JUN 02   The first reclassification of IPC codes now complete in
                   INPADOC
 NEWS 11  JUN 26   TULSA/TULSA2 reloaded and enhanced with new search and
                   and display fields
 NEWS 12  JUN 28   Price changes in full-text patent databases EPFULL and PCTFULL
 NEWS 13  JUl 11   CHEMSAFE reloaded and enhanced
 NEWS 14  JUl 14   FSTA enhanced with Japanese patents
 NEWS 15  JUl 19   Coverage of Research Disclosure reinstated in DWPI

 NEWS EXPRESS  JUNE 30 CURRENT WINDOWS VERSION IS V8.01b, CURRENT
               MACINTOSH VERSION IS V6.0c(ENG) AND V6.0Jc(JP),
               AND CURRENT DISCOVER FILE IS DATED 26 JUNE 2006.

 NEWS HOURS    STN Operating Hours Plus Help Desk Availability
 NEWS LOGIN    Welcome Banner and News Items
 NEWS IPC8     For general information regarding STN implementation of IPC 8
 NEWS X25      X.25 communication option no longer available

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FILE 'HOME' ENTERED AT 08:12:06 ON 06 AUG 2006

=> index bioscience medicine
FILE 'DRUGMONOG' ACCESS NOT AUTHORIZED
COST IN U.S. DOLLARS                                SINCE FILE      TOTAL
                                                       ENTRY    SESSION
FULL ESTIMATED COST                                     0.21       0.21

INDEX 'ADISCTI, ADISINSIGHT, ADISNEWS, AGRICOLA, ANABSTR, ANTE, AQUALINE,
       AQUASCI, BIOENG, BIOSIS, BIOTECHABS, BIOTECHDS, BIOTECHNO, CABA, CAPLUS,
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      CEABA-VTB, CIN, CONFSCI, CROPB, CROPU, DDFB, DDFU, DGENE, DISSABS, DRUGB,
      DRUGMONOG2, DRUGU, EMBAL, EMBASE, ...' ENTERED AT 08:12:22 ON 06 AUG 2006

71 FILES IN THE FILE LIST IN STNINDEX

Enter SET DETAIL ON to see search term postings or to view
search error messages that display as 0* with SET DETAIL OFF.

=> s glucosamin?(s)(synthe? or produc? or proce? or manufac? or biosynth?)
         21   FILE ADISCTI
         25   FILE ADISINSIGHT
         30   FILE ADISNEWS
        344   FILE AGRICOLA
         77   FILE ANABSTR
          6   FILE ANTE
          7   FILE AQUALINE
        184   FILE AQUASCI
        392   FILE BIOENG
       2967   FILE BIOSIS
        633   FILE BIOTECHABS
  11 FILES SEARCHED...
        633   FILE BIOTECHDS
       1987   FILE BIOTECHNO
       1095   FILE CABA
       4407   FILE CAPLUS
        103   FILE CEABA-VTB
         37   FILE CIN
  17 FILES SEARCHED...
         23   FILE CONFSCI
          3   FILE CROPB
         59   FILE CROPU
        201   FILE DDFB
        426   FILE DDFU
       3267   FILE DGENE
  23 FILES SEARCHED...
        327   FILE DISSABS
        201   FILE DRUGB
        804   FILE DRUGU
         29   FILE EMBAL
       2084   FILE EMBASE
       1710   FILE ESBIOBASE
         18   FILE FOMAD
          1   FILE FOREGE
        197   FILE FROSTI
  33 FILES SEARCHED...
        216   FILE FSTA
       1161   FILE GENBANK
         15   FILE HEALSAFE
        658   FILE IFIPAT
          5   FILE IMSDRUGNEWS
          9   FILE IMSRESEARCH
        546   FILE JICST-EPLUS
         35   FILE KOSMET
       1570   FILE LIFESCI
       2251   FILE MEDLINE
         39   FILE NTIS
  45 FILES SEARCHED...
         66   FILE NUTRACEUT
         45   FILE OCEAN
       1594   FILE PASCAL
  48 FILES SEARCHED...
          9   FILE PHAR
          4   FILE PHARMAML
         88   FILE PHIN
        986   FILE PROMT
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          12   FILE PROUSDDR
           7   FILE RDISCLOSURE
        1788   FILE SCISEARCH
          11   FILE SYNTHLINE
        1158   FILE TOXCENTER
  60 FILES SEARCHED...
        4724   FILE USPATFULL
         483   FILE USPAT2
           2   FILE VETB
          41   FILE VETU
          22   FILE WATER
         871   FILE WPIDS
  66 FILES SEARCHED...
           7   FILE WPIFV
         871   FILE WPINDEX
  68 FILES SEARCHED...
          48   FILE IPA
          10   FILE NAPRALERT
         506   FILE NLDB

  66 FILES HAVE ONE OR MORE ANSWERS,   71 FILES SEARCHED IN STNINDEX

L1   QUE GLUCOSAMIN?(S)(SYNTHE? OR PRODUC? OR PROCE? OR MANUFAC? OR BIOSYNTH?)

=> d rank
F1        4724   USPATFULL
F2        4407   CAPLUS
F3        3267   DGENE
F4        2967   BIOSIS
F5        2251   MEDLINE
F6        2084   EMBASE
F7        1987   BIOTECHNO
F8        1788   SCISEARCH
F9        1710   ESBIOBASE
F10       1594   PASCAL
F11       1570   LIFESCI
F12       1161   GENBANK
F13       1158   TOXCENTER
F14       1095   CABA
F15        986   PROMT
F16        871   WPIDS
F17        871   WPINDEX
F18        804   DRUGU
F19        658   IFIPAT
F20        633   BIOTECHABS
F21        633   BIOTECHDS
F22        546   JICST-EPLUS
F23        506   NLDB
F24        483   USPAT2
F25        426   DDFU
F26        392   BIOENG
F27        344   AGRICOLA
F28        327   DISSABS
F29        216   FSTA
F30        201   DDFB
F31        201   DRUGB
F32        197   FROSTI
F33        184   AQUASCI
F34        103   CEABA-VTB
F35         88   PHIN
F36         77   ANABSTR
F37         66   NUTRACEUT
F38         59   CROPU
F39         48   IPA
F40         45   OCEAN
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F41          41   VETU
F42          39   NTIS
F43          37   CIN
F44          35   KOSMET
F45          30   ADISNEWS
F46          29   EMBAL
F47          25   ADISINSIGHT
F48          23   CONFSCI
F49          22   WATER
F50          21   ADISCTI
F51          18   FOMAD
F52          15   HEALSAFE
F53          12   PROUSDDR
F54          11   SYNTHLINE
F55          10   NAPRALERT
F56           9   IMSRESEARCH
F57           9   PHAR
F58           7   AQUALINE
F59           7   RDISCLOSURE
F60           7   WPIFV
F61           6   ANTE
F62           5   IMSDRUGNEWS
F63           4   PHARMAML
F64           3   CROPB
F65           2   VETB
F66           1   FOREGE


=> file f1-f2, f4-f11
COST IN U.S. DOLLARS                                SINCE FILE      TOTAL
                                                         ENTRY    SESSION
FULL ESTIMATED COST                                       7.93       8.14

FILE 'USPATFULL' ENTERED AT 08:20:19 ON 06 AUG 2006
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FILE 'LIFESCI' ENTERED AT 08:20:19 ON 06 AUG 2006
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=> s glucosamin?(s)(synthe? or produc? or proce? or manufac? or biosynth?)
   7 FILES SEARCHED...
   9 FILES SEARCHED...
L2       25082 GLUCOSAMIN?(S)(SYNTHE? OR PRODUC? OR PROCE? OR MANUFAC? OR BIOSY
               NTH?)

=> s l2(s)(acetyltransferas?)
L3         225 L2(S)(ACETYLTRANSFERAS?)

=> s l3(s)phosphat?
L4         134 L3(S) PHOSPHAT?

=> dup rem l4
PROCESSING COMPLETED FOR L4
L5          95 DUP REM L4 (39 DUPLICATES REMOVED)

=> s l5(s)(synthetas? or synthas?)
PROXIMITY OPERATOR LEVEL NOT CONSISTENT WITH
FIELD CODE - 'AND' OPERATOR ASSUMED 'L42(S)(SYNTHETAS'
PROXIMITY OPERATOR LEVEL NOT CONSISTENT WITH
FIELD CODE - 'AND' OPERATOR ASSUMED 'L44(S)(SYNTHETAS'
PROXIMITY OPERATOR LEVEL NOT CONSISTENT WITH
FIELD CODE - 'AND' OPERATOR ASSUMED 'L48(S)(SYNTHETAS'
L6          64 L5(S)(SYNTHETAS? OR SYNTHAS?)

=> d ti l6 1-64

L6   ANSWER 1 OF 64  USPATFULL on STN
TI     Sequence-determined DNA fragments and corresponding polypeptides encoded
       thereby

L6   ANSWER 2 OF 64  USPATFULL on STN
TI     Nucleic acid and amino acid sequences relating to Enterobacter cloacae
       for diagnostics and therapeutics

L6   ANSWER 3 OF 64  USPATFULL on STN
TI     Process and materials for production of glucosamine

L6   ANSWER 4 OF 64  USPATFULL on STN
TI     Listeria monocytogenes genome, polypeptides and uses

L6   ANSWER 5 OF 64  USPATFULL on STN
TI     Complete genome and protein sequence of the hyperthermophile
       methanopyrus kandleri av19 and monophyly of archael methanogens and
       methods of use thereof

L6   ANSWER 6 OF 64  USPATFULL on STN
TI     Outcome prediction and risk classification in childhood leukemia

L6   ANSWER 7 OF 64  USPATFULL on STN
TI     System for regulating in vivo the expression of a transgene by
       conditional inhibition

L6   ANSWER 8 OF 64  USPATFULL on STN
TI     Gene expression profiling of colon cancer with DNA arrays

L6   ANSWER 9 OF 64  USPATFULL on STN
TI     Genetic networks regulated by attenuated GH/IGF1 signaling and caloric
       restriction

L6   ANSWER 10 OF 64  USPATFULL on STN
TI     Genes of an otitis media isolate of haemophilus influenzae
```

L6 ANSWER 11 OF 64 USPATFULL on STN
TI Signatures of ER status in breast cancer

L6 ANSWER 12 OF 64 USPATFULL on STN
TI Methods and apparatus for gel-free qualitative and quantitative proteome analysis, and uses therefore

L6 ANSWER 13 OF 64 USPATFULL on STN
TI Methods for monitoring multiple gene expression

L6 ANSWER 14 OF 64 USPATFULL on STN
TI Lactobacillus acidophilus nucleic acid sequences encoding cell surface protein homologues and uses therefore

L6 ANSWER 15 OF 64 USPATFULL on STN
TI Breast cancer signatures

L6 ANSWER 16 OF 64 USPATFULL on STN
TI Acyl-nucleotide probes and methods of their synthesis and use in proteomic analysis

L6 ANSWER 17 OF 64 USPATFULL on STN
TI Metabolic engineering for enhanced production of chitin and chitosan in microorganisms

L6 ANSWER 18 OF 64 USPATFULL on STN
TI Processes for producing sugar nucleotides and complex carbohydrates

L6 ANSWER 19 OF 64 USPATFULL on STN
TI Translational profiling

L6 ANSWER 20 OF 64 USPATFULL on STN
TI Polypeptides from Chlamydia pneumoniae and their use in the diagnosis, prevention and treatment of disease

L6 ANSWER 21 OF 64 USPATFULL on STN
TI Methods for monitoring multiple gene expression

L6 ANSWER 22 OF 64 USPATFULL on STN
TI Methods of screening test compounds using GADD34L, an eIF2alpha-specific phosphatase subunit

L6 ANSWER 23 OF 64 USPATFULL on STN
TI Nucleic acid sequences relating to Candida albicans for diagnostics and therapeutics

L6 ANSWER 24 OF 64 USPATFULL on STN
TI Process and materials for production of glucosamine and N-acetylglucosamine

L6 ANSWER 25 OF 64 USPATFULL on STN
TI Nucleotide sequences of moraxella catarrhalis genome

L6 ANSWER 26 OF 64 USPATFULL on STN
TI Novel proteins and nucleic acids encoding same

L6 ANSWER 27 OF 64 USPATFULL on STN
TI Gene expression in bladder tumors

L6 ANSWER 28 OF 64 USPATFULL on STN
TI Myc targets

L6 ANSWER 29 OF 64 USPATFULL on STN
TI Rice promoters for regulation of plant expression

L6 ANSWER 30 OF 64 USPATFULL on STN
TI Chlamydia pneumoniae polynucleotides and uses thereof

L6 ANSWER 31 OF 64 USPATFULL on STN
TI Methods and apparatuses for gel-free qualitative and quantitative proteome analysis, and uses therefore

L6 ANSWER 32 OF 64 USPATFULL on STN
TI Methods of diagnosis of ovarian cancer, compositions and methods of screening for modulators of ovarian cancer

L6 ANSWER 33 OF 64 USPATFULL on STN
TI Rapid quantitative analysis of proteins or protein function in complex mixtures

L6 ANSWER 34 OF 64 USPATFULL on STN
TI Compostions and methods for modeling Saccharomyces cerevisiae metabolism

L6 ANSWER 35 OF 64 USPATFULL on STN
TI Pre-and post therapy gene expression profiling to identify drug targets

L6 ANSWER 36 OF 64 USPATFULL on STN
TI Compositions and methods for modeling bacillus subtilis metabolism

L6 ANSWER 37 OF 64 USPATFULL on STN
TI Genostics

L6 ANSWER 38 OF 64 USPATFULL on STN
TI Methods for the identification of fungal glucose utilization inhibitors and antifungal agents

L6 ANSWER 39 OF 64 USPATFULL on STN
TI Nucleic acids encoding 3-ketoacyl-ACP reductase from Moraxella catarrahalis

L6 ANSWER 40 OF 64 USPATFULL on STN
TI Method for identifying helicobacter antigens

L6 ANSWER 41 OF 64 USPATFULL on STN
TI Methods and compositions for genetically modifying primate bone marrow cells

L6 ANSWER 42 OF 64 USPATFULL on STN
TI Methods for predicting functional and structural properties of polypeptides using sequence models

L6 ANSWER 43 OF 64 USPATFULL on STN
TI Libraries of expressible gene sequences

L6 ANSWER 44 OF 64 USPATFULL on STN
TI Staphylococcus aureus polynucleotides and sequences

L6 ANSWER 45 OF 64 USPATFULL on STN
TI Nucleic acid and amino acid sequences relating to mycobacterium tuberculosis and leprae for diagnostics and therapeutics

L6 ANSWER 46 OF 64 USPATFULL on STN
TI Nucleic acid and amino acid sequences relating to Acinetobacter baumannii for diagnostics and therapeutics

L6 ANSWER 47 OF 64 USPATFULL on STN
TI Chlamydia pneumoniae polynucleotides and uses thereof

L6 ANSWER 48 OF 64 USPATFULL on STN

TI Identification of modulatory molecules using inducible promoters

L6 ANSWER 49 OF 64 USPATFULL on STN
TI Libraries of expressible gene sequences

L6 ANSWER 50 OF 64 USPATFULL on STN
TI Method of identifying renalgenerative agents using differential gene expression

L6 ANSWER 51 OF 64 USPATFULL on STN
TI Process and materials for production of glucosamine

L6 ANSWER 52 OF 64 USPATFULL on STN
TI Methods of screening test substances for treating or preventing diseases involving an oxidative stress

L6 ANSWER 53 OF 64 USPATFULL on STN
TI Methods for the identification of antimicrobial compounds

L6 ANSWER 54 OF 64 USPATFULL on STN
TI PROCESS FOR PRODUCTION OF N-GLUCOSAMINE

L6 ANSWER 55 OF 64 USPATFULL on STN
TI ENTEROCOCCUS FAECALIS POLYNUCLEOTIDES AND POLYPEPTIDES

L6 ANSWER 56 OF 64 USPATFULL on STN
TI Rapid quantitative analysis of proteins or protein function in complex mixtures

L6 ANSWER 57 OF 64 USPATFULL on STN
TI PROCESSES FOR PRODUCING SUGAR NUCLEOTIDES AND COMPLEX CARBOHYDRATES

L6 ANSWER 58 OF 64 USPATFULL on STN
TI Process and materials for production of glucosamine

L6 ANSWER 59 OF 64 USPATFULL on STN
TI Processes for producing sugar nucleotides and complex carbohydrates

L6 ANSWER 60 OF 64 USPATFULL on STN
TI Expressed sequences of arabidopsis thaliana

L6 ANSWER 61 OF 64 CAPLUS COPYRIGHT 2006 ACS on STN
TI Myxospore coat synthesis in Myxococcus xanthus: enzymes associated with uridine 5'-diphosphate-N-acetylgalactosamine formation during myxospore development

L6 ANSWER 62 OF 64 BIOTECHNO COPYRIGHT 2006 Elsevier Science B.V. on STN
TI Subcellular distribution of glycolyltransferases in rodent liver and their significance in special reference to the synthesis of N-glycolylneuraminic acid

L6 ANSWER 63 OF 64 Elsevier BIOBASE COPYRIGHT 2006 Elsevier Science B.V. on STN
TI Mosquito glucosamine-6-phosphate N-acetyltransferase: cDNA, gene structure and enzyme kinetics

L6 ANSWER 64 OF 64 LIFESCI COPYRIGHT 2006 CSA on STN
TI Purification and properties of acetyl-CoA: Glucosamine 6-phosphate N-acetyltransferase from rat liver.

=> d l6 ibib abs 6 17 24 51 54 58

L6 ANSWER 6 OF 64 USPATFULL on STN
ACCESSION NUMBER: 2006:74111 USPATFULL

TITLE: Outcome prediction and risk classification in childhood leukemia

INVENTOR(S): Willman, Cheryl L., Albuquerque, NM, UNITED STATES
Helman, Paul, Albuquerque, NM, UNITED STATES
Veroff, Robert, Albuquerque, NM, UNITED STATES
Mosquera-Caro, Monica, Albuquerque, NM, UNITED STATES
Davidson, George S., Albuquerque, NM, UNITED STATES
Martin, Shawn B., Albuquerque, NM, UNITED STATES
Atlas, Susan R., Albuquerque, NM, UNITED STATES
Andries, Erik, Rio Rancho, NM, UNITED STATES
Kang, Huining, Albuquerque, NM, UNITED STATES
Shuster, Jonathan J., Gainesville, FL, UNITED STATES
Wang, Xuefei, Albuquerque, NM, UNITED STATES
Harvey, Richard C., Placitas, NM, UNITED STATES
Haaland, David M., Albuquerque, NM, UNITED STATES
Potter, Jeffrey W., Albuquerque, NM, UNITED STATES

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 2006063156	A1	20060323	
APPLICATION INFO.:	US 2003-729895	A1	20031205	(10)

	NUMBER	DATE	
PRIORITY INFORMATION:	US 2002-432064P	20021206	(60)
	US 2002-432077P	20021206	(60)
	US 2002-432078P	20021206	(60)
	US 2003-510904P	20031014	(60)
	US 2003-510968P	20031014	(60)
	US 2003-527610P	20031205	(60)

DOCUMENT TYPE: Utility
FILE SEGMENT: APPLICATION
LEGAL REPRESENTATIVE: COLEMAN SUDOL SAPONE, P.C., 714 COLORADO AVENUE, BRIDGE PORT, CT, 06605-1601, US
NUMBER OF CLAIMS: 42
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 23 Drawing Page(s)
LINE COUNT: 12227
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Genes and gene expression profiles useful for predicting outcome, risk classification, cytogenetics and/or etiology in pediatric acute lymphoblastic leukemia (ALL). OPAL1 is a novel gene associated with outcome and, along with other newly identified genes, represent a novel therapeutic targets.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L6 ANSWER 17 OF 64 USPATFULL on STN
ACCESSION NUMBER: 2005:49939 USPATFULL
TITLE: Metabolic engineering for enhanced production of chitin and chitosan in microorganisms

INVENTOR(S): Deng, Ming-De, Manitowoc, WI, UNITED STATES
McMullin, Thomas W., Manitowoc, WI, UNITED STATES
Grund, Alan D., Manitowoc, WI, UNITED STATES

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 2005042735	A1	20050224	
APPLICATION INFO.:	US 2004-823397	A1	20040412	(10)

	NUMBER	DATE	
PRIORITY INFORMATION:	US 2003-462087P	20030411	(60)

DOCUMENT TYPE: Utility
FILE SEGMENT: APPLICATION

LEGAL REPRESENTATIVE: SHERIDAN ROSS PC, 1560 BROADWAY, SUITE 1200, DENVER, CO, 80202
NUMBER OF CLAIMS: 35
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 1 Drawing Page(s)
LINE COUNT: 6323
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB Disclosed is a fermentation method for the production of commercially useful amounts of chitin and/or chitosan by a biosynthetic process. Also disclosed are genetically modified microorganisms useful in such a method and a microbial biomass containing chitin and/or chitosan produced by such a method.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L6 ANSWER 24 OF 64 USPATFULL on STN
ACCESSION NUMBER: 2004:120566 USPATFULL
TITLE: Process and materials for production of glucosamine and N-acetylglucosamine
INVENTOR(S): Deng, Ming-De, Manitowoc, WI, UNITED STATES
Angerer, J. David, Hockessin, DE, UNITED STATES
Cyron, Don, Lincoln University, PA, UNITED STATES
Grund, Alan D., Manitowoc, WI, UNITED STATES
Jerrell, Thomas A., JR., Manitowoc, WI, UNITED STATES
Leanna, Candice, Green Bay, WI, UNITED STATES
Mathre, Owen, Wilmington, DE, UNITED STATES
Rosson, Reinhardt, Manitowoc, WI, UNITED STATES
Running, Jeff, Manitowoc, WI, UNITED STATES
Severson, Dave, Two Rivers, WI, UNITED STATES
Song, Linsheng, Manitowoc, WI, UNITED STATES
Wassink, Sarah, Sheboygan, WI, UNITED STATES

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 2004091976	A1	20040513
APPLICATION INFO.:	US 2003-612779	A1	20030701 (10)

	NUMBER	DATE
PRIORITY INFORMATION:	US 2002-393348P	20020701 (60)

DOCUMENT TYPE: Utility
FILE SEGMENT: APPLICATION
LEGAL REPRESENTATIVE: SHERIDAN ROSS PC, 1560 BROADWAY, SUITE 1200, DENVER, CO, 80202
NUMBER OF CLAIMS: 212
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 25 Drawing Page(s)
LINE COUNT: 15039
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB A biosynthetic method for producing glucosamine and N-acetylglucosamine is disclosed. Such a method includes the fermentation of a genetically modified microorganism to produce glucosamine and/or N-acetylglucosamine. Also disclosed are genetically modified microorganisms that are useful for producing glucosamine and N-acetylglucosamine. In addition, methods of recovering N-acetylglucosamine that has been produced by a fermentation process, including methods that result in N-acetylglucosamine of high purity, are described. Also disclosed is a method to produce glucosamine from N-acetylglucosamine.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L6 ANSWER 51 OF 64 USPATFULL on STN
ACCESSION NUMBER: 2003:64818 USPATFULL
TITLE: Process and materials for production of glucosamine

INVENTOR(S): Berry, Alan, Manitowoc, WI, UNITED STATES
Burlingame, Richard P., Manitowoc, WI, UNITED STATES
Millis, James R., Kohler, WI, UNITED STATES
PATENT ASSIGNEE(S): Arkion Life Sciences LLC (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 2003044939	A1	20030306
APPLICATION INFO.:	US 2001-24460	A1	20011217 (10)

RELATED APPLN. INFO.: Continuation of Ser. No. US 1998-115475, filed on 15 Jul 1998, GRANTED, Pat. No. US 6372457
Continuation-in-part of Ser. No. WO 1998-US800, filed on 14 Jan 1998, UNKNOWN

	NUMBER	DATE
PRIORITY INFORMATION:	US 1997-35494P	19970114 (60)

DOCUMENT TYPE: Utility
FILE SEGMENT: APPLICATION
LEGAL REPRESENTATIVE: SHERIDAN ROSS PC, 1560 BROADWAY, SUITE 1200, DENVER, CO, 80202
NUMBER OF CLAIMS: 60
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 16 Drawing Page(s)
LINE COUNT: 4958
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB The present invention relates to a method and materials for producing glucosamine by fermentation of a genetically modified microorganism. Included in the present invention are genetically modified microorganisms useful in the present method for producing glucsamine, as well as recombinant nucleic acid molecules and the proteins produces by such recombinant nucleic acid molecules.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L6 ANSWER 54 OF 64 USPATFULL on STN
ACCESSION NUMBER: 2002:287596 USPATFULL
TITLE: PROCESS FOR PRODUCTION OF N-GLUCOSAMINE
INVENTOR(S): BERRY, ALAN, BLOOMFIELD, NJ, UNITED STATES
BURLINGAME, RICHARD P., MANITOWOC, WI, UNITED STATES
MILLIS, JAMES R., KOHLER, WI, UNITED STATES

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 2002160459	A1	20021031
APPLICATION INFO.:	US 1999-341600	A1	19990915 (9)
	WO 1998-US800		19980114

DOCUMENT TYPE: Utility
FILE SEGMENT: APPLICATION
LEGAL REPRESENTATIVE: SHERIDAN ROSS PC, 1560 BROADWAY, SUITE 1200, DENVER, CO, 80202
NUMBER OF CLAIMS: 39
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 9 Drawing Page(s)
LINE COUNT: 2181
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB The present invention relates to a method for producing N-glucosamine by fermentation of a genetically modified microorganism.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L6 ANSWER 58 OF 64 USPATFULL on STN
ACCESSION NUMBER: 2002:81241 USPATFULL
TITLE: Process and materials for production of glucosamine
INVENTOR(S): Berry, Alan, Manitowoc, WI, United States

Burlingame, Richard P., Manitowoc, WI, United States
Millis, James R., Kohler, WI, United States
PATENT ASSIGNEE(S): Arkion Life Sciences LLC, Wilmington, DE, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6372457	B1	20020416
APPLICATION INFO.:	US 1998-115475		19980715 (9)

RELATED APPLN. INFO.: Continuation-in-part of Ser. No. WO 1998-US800, filed on 14 Jan 1998

	NUMBER	DATE
PRIORITY INFORMATION:	US 1997-35494P	19970114 (60)

DOCUMENT TYPE: Utility
FILE SEGMENT: GRANTED
PRIMARY EXAMINER: Nashed, Nashaat T.
ASSISTANT EXAMINER: Fronda, Christian L
LEGAL REPRESENTATIVE: Sheridan Ross P.C.
NUMBER OF CLAIMS: 59
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 21 Drawing Figure(s); 21 Drawing Page(s)
LINE COUNT: 4835
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB The present invention relates to a method and materials for producing glucosamine by fermentation of a genetically modified microorganism. Included in the present invention are genetically modified microorganisms useful in the present method for producing glucsamine, as well as recombinant nucleic acid molecules and the proteins produces by such recombinant nucleic acid molecules.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

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L6 ANSWER 3 OF 64 USPATFULL on STN
ACCESSION NUMBER: 2006:111141 USPATFULL
TITLE: Process and materials for production of glucosamine
INVENTOR(S): Berry, Alan, Manitowoc, WI, UNITED STATES
Burlingame, Richard P., Manitowoc, WI, UNITED STATES
Millis, James R., Kohler, WI, UNITED STATES
PATENT ASSIGNEE(S): Arkion Life Sciences LLC d/b/a Bio-Technical Resources, Manitowoc, WI, UNITED STATES (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 2006094085	A1	20060504
APPLICATION INFO.:	US 2005-245473	A1	20051005 (11)

RELATED APPLN. INFO.: Continuation of Ser. No. US 2001-24460, filed on 17 Dec 2001, ABANDONED Continuation of Ser. No. US 1998-115475, filed on 15 Jul 1998, GRANTED, Pat. No. US 6372457 Continuation-in-part of Ser. No. WO 1998-US800, filed on 14 Jan 1998, PENDING

	NUMBER	DATE
PRIORITY INFORMATION:	US 1997-35494P	19970114 (60)

DOCUMENT TYPE: Utility
FILE SEGMENT: APPLICATION
LEGAL REPRESENTATIVE: SHERIDAN ROSS PC, 1560 BROADWAY, SUITE 1200, DENVER, CO, 80202, US
NUMBER OF CLAIMS: 27
EXEMPLARY CLAIM: 1

NUMBER OF DRAWINGS: 16 Drawing Page(s)
LINE COUNT: 4706
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB The present invention relates to a method and materials for producing glucosamine by fermentation of a genetically modified microorganism. Included in the present invention are genetically modified microorganisms useful in the present method for producing glucsamine, as well as recombinant nucleic acid molecules and the proteins produces by such recombinant nucleic acid molecules.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

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(FILE 'HOME' ENTERED AT 08:12:06 ON 06 AUG 2006)

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              48   FILE IPA
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     FILE USPATFULL
     FILE COVERS 1971 TO PATENT PUBLICATION DATE: 3 Aug 2006 (20060803/PD)
     FILE LAST UPDATED: 3 Aug 2006 (20060803/ED)
     HIGHEST GRANTED PATENT NUMBER: US7086090
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     CA INDEXING IS CURRENT THROUGH 1 Aug 2006 (20060801/UPCA)
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Metabolic engineering of *Escherichia coli* for industrial production of glucosamine and *N*-acetylglucosamine

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Abstract

Glucosamine and *N*-acetylglucosamine are currently produced by extraction and acid hydrolysis of chitin from shellfish waste. Production could be limited by the amount of raw material available and the product potentially carries the risk of shellfish protein contamination. *Escherichia coli* was modified by metabolic engineering to develop a fermentation process. Over-expression of glucosamine synthase (GlmS) and inactivation of catabolic genes increased glucosamine production by 15 fold, reaching 60 mg l^{-1}. Since GlmS is strongly inhibited by glucosamine-6-P, GlmS variants were generated via error-prone PCR and screened. Over-expression of an improved enzyme led to a glucosamine titer of 17 g l^{-1}. Rapid degradation of glucosamine and inhibitory effects of glucosamine and its degradation products on host cells limited further improvement. An alternative fermentation product, *N*-acetylglucosamine, is stable, non-inhibitory to the host and readily hydrolyzed to glucosamine under acidic conditions. Therefore, the glucosamine pathway was extended to *N*-acetylglucosamine by over-expressing a heterologous glucosamine-6-P *N*-acetyltransferase. Using a simple and low-cost fermentation process developed for this strain, over 110 g l^{-1} of *N*-acetylglucosamine was produced.

Keywords: Glucosamine; *N*-acetylglucosamine; *Escherichia coli*; *glmS* gene; *GNA1* gene; Metabolic engineering; Fermentation; Lactose induction

1. Introduction

Glucosamine (GlcN, 2-amino-2-deoxy-D-glucose) and its derivative *N*-acetylglucosamine (GlcNAc, 2-acetamido-2-deoxy-D-glucose) are synthesized in all organisms, including bacteria, yeast, filamentous fungi, plants and animals. In humans, GlcN and GlcNAc are precursors of the disaccharide units in glycosaminoglycans (such as hyaluronic acid, chondroitin sulfate and keratan sulfate), which are necessary to repair and maintain healthy cartilage and joint function. Clinical trials with GlcN for treatment of arthritis started in the early 1980s. Since then GlcN has been increasingly used as a dietary supplement (Hungerford and Jones, 2003; Towheed, 2003). It is estimated that 33 million people suffer from osteoarthritis in the United States. The National Institutes of Health (NIH) is conducting a Phase III clinical trial, a multiple-site, double-blind randomized study on the effectiveness of GlcN for treatment of knee osteoarthritis (NIH GlcN/Chondroitin Arthritis Intervention Trial (GAIT); http://clinicaltrials.gov/show/NCT00032890). Results of the study are expected to be published in November 2005.

Currently, GlcN is produced by acid hydrolysis of chitin (a linear polymer of GlcNAc) extracted from crab

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and shrimp shells. Concentrated hydrochloric acid breaks down the polymer and deacetylates GlcNAc to form GlcN (Novikov and Ivanov, 1997). GlcNAc is produced by chemical acetylation of GlcN using acetic anhydride (He et al., 2001). GlcN production could become limited by variable raw material supply as the demand continues to increase. Moreover, GlcN from shellfish may not be suitable for people with shellfish allergies. Some filamentous fungi contain chitosan (a linear co-polymer of GlcN and GlcNAc, with GlcN accounting for over 65%) in their cell walls (Velichkov and Georgiev, 1991). The amount of chitosan in fungal biomass is low, generally less than 25% of dry cell weight. Current economics limit GlcN production to the use of fungal biomass from fermentation processes dedicated to citric acid or other primary products.

In the present report, a new microbial fermentation for production of high-quality and low-cost GlcN and GlcNAc was developed. The strategy to develop an economically viable fermentation process was to genetically engineer a high-performance production strain. Pathways for the synthesis and metabolism of GlcN are well characterized in the bacterium *Escherichia coli* and other organisms (Plumbridge, 1995; Plumbridge and Pellegrini, 2004). As shown in Fig. 1, GlcN synthase (encoded by *glmS*) converts fructose-6-phosphate (fructose-6-P) to GlcN-6-P using glutamine as an amino donor. For the synthesis of peptidoglycan and lipopolysaccharide, essential components of the cell wall of gram-negative bacteria, GlcN-6-P is converted to GlcN-1-P by phosphoglucosamine mutase (encoded by *glmM*). The product is further converted by a bifunctional enzyme, GlcN-1-P *N*-acetyltransferase/GlcNAc-1-P uridyltransferase (encoded by *glmU*), to GlcNAc-1-P and then to UDP-GlcNAc.

GlcN and GlcNAc also serve as alternative carbon and nitrogen sources. GlcN is transported and phosphorylated by a mannose transporter (II^{Man}, encoded by the operon *manXYZ*) and the glucose transporter (II^{Glc}, encoded by *ptsG*). GlcNAc is transported and phosphorylated by the mannose transporter and a GlcNAc-specific transporter (II^{NAG}, encoded by *nagE*). GlcNAc-6-P deacetylase (encoded by *nagA*) converts GlcNAc-6-P to GlcN-6-P, which is further converted to fructose-6-P by GlcN-6-P deaminase (encoded by *nagB*). In the *E. coli* genome, *glmS* and *glmU* constitute the operon *glmUS* while *nagA*, *-B*, *-C*, *-D* and *-E* form a regulon (Plumbridge, 1995). The *nagC* gene encodes a regulatory protein that functions as a repressor of the *nag* regulon as well as both an activator and repressor of the *glmUS* operon. The function of *nagD* is not known but is likely related to amino sugar metabolism, as it resides in the *nag* regulon.

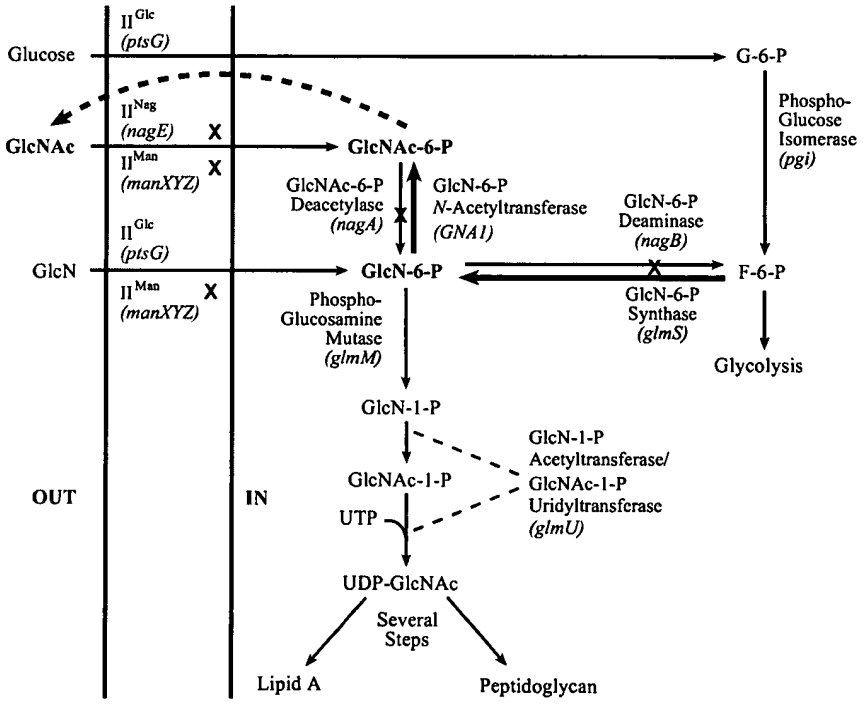

Fig. 1. Pathway engineering for GlcNAc production in recombinant *E. coli*. Crosses indicate metabolic flux blocked by gene inactivation or deletion. Thicker lines indicate introduction and/or increase of the metabolic flux by gene over-expression. Dephosphorylation and secretion of GlcNAc-6-P are indicated as a dotted line. G-6-P: glucose-6-phosphate, F-6-P: fructose-6-phosphate, GlcN: glucosamine, GlcNAc: *N*-acetylglucosamine, II^{Glc}: glucose transporter, II^{NAG}: GlcNAc-specific transporter, II^{Man}: mannose transporter.

Both the synthetic and degradative pathways for GlcN and GlcNAc are tightly regulated. In the present work, metabolic engineering was used to develop *E. coli* strains for production by fermentation of the monomers of GlcN and GlcNAc. Strain and process optimization led to the development of an economically competitive process.

2. Materials and methods

2.1. Development of the E. coli strain 7101-17(DE3)

E. coli K-12 (ATCC 25947, a derivative of W3110) (Bachmann, 1996) was used as the metabolic engineering host to produce GlcN and GlcNAc. The strain was modified by P1 phage transduction (Miller, 1992) to introduce a mutation or deletion in operons involved in the transport and catabolism of GlcN and GlcNAc. A mutation in *manXYZ* was introduced using phage grown on *E. coli* strain IBPC566 (courtesy of Dr. Plumbridge, Institut de Biologie Physico-Chimique, Paris, France) which contained a tetracycline resistance determinant (tet^R from transposon Tn10) linked to the *manXYZ* mutation (designated *manXYZ8 zdj-225*::Tn10) (Plumbridge, 1990). The resulting strain 7101-6 grew poorly on mannose and failed to grow with GlcN as the sole carbon source. In *E. coli* strain IBPC590 (courtesy of Dr. Plumbridge) the entire *nagBACD* operon and a portion of *nagE* was replaced by a tet^R determinant (Plumbridge, 1992). In order to use tetracycline selection to introduce the *nag* deletion, strain 7101-6 was plated onto TCS medium which selects for tetracycline-sensitive mutants as described by Maloy and Nunn (1981). Southern blot hybridization showed that the resistance was lost by the deletion of the tet^R marker in strain 7101-13. The $\Delta nag::tet^R$ mutation in IBPC590 was introduced by P1 phage transduction into 7101-13 to generate strain 7101-17 (*manXYZ8*, $\Delta nag::tet^R$). The defective λDE3 prophage containing a T7 RNA polymerase gene under *lacUV5* promoter control was introduced into 7101-17 using the λDE3 Lysogenization Kit (Novagen, Inc. Madison, WI), generating a T7 expression host, 7101-17(DE3).

2.2. Cloning and integration of the E. coli glmS gene

The *E. coli glmS* gene (Walker et al., 1984) was amplified from *E. coli* genomic DNA by Polymerase Chain Reaction (PCR). The forward primer Up1 (5′-CGG TCT CCC ATG TGT GGA ATT GTT GGC GC-3′) contained sequences corresponding to the first 22 nucleotides of the *glmS* coding sequence and a *Bsa*I restriction site. The reverse primer Lo8 (5′-CTC TAG AGC GTT GAT ATT CAG TCA ATT ACA AAC A-3′) contained sequences corresponding to positions between 145 and 177 bases downstream of the *glmS* stop codon and an *Xba*I site. PCR amplification generated a *Bsa*I–*Xba*I fragment containing the *glmS* gene with 177 bp of downstream sequence, including the *glmS* terminator, between positions 6 and 43 following the stop codon (Gay et al., 1986). The PCR product was cloned as a blunt-end fragment into pPCR-Script™Amp SK(+) (Stratagene Cloning System, La Jolla, CA). In the recombinant plasmid a unique *Sac*I restriction site was located 215 bp downstream of the *glmS*.stop codon. The *glmS* sequence was isolated as a *Bsa*I–*Sac*I fragment and cloned between *Nco*I and *Sac*I sites of the expression vector pET24d(+) (Novagen) to generate plasmid pKLN23-23. Cloning in this manner resulted in a *T7lac–glmS* expression cassette containing the *glmS* terminator.

The *T7lac–glmS* cassette was integrated into the *E. coli* chromosome in 7101-17(DE3) by a multistep process. First, the cassette was excised from pKLN23-23 as a *Bgl*II–*Hin*dIII fragment by a partial digestion with *Bgl*II and a complete digestion with *Hin*dIII. The plasmid contained three *Bgl*II sites, one located approximately 20 bp upstream of the T7 promoter, and two in *glmS*. The unique *Hin*dIII site is located 224 bp downstream of the *glmS* stop codon. The expression cassette was cloned between the unique *Bam*HI and *Hin*dIII sites of plasmid pBRINT–Cm, an integrative vector containing a chloramphenicol selection marker (Cm) at the *lacZ* deletion site (Balbas et al., 1996). This resulted in plasmid pKLN23-28 containing the *T7lac–glmS–Cm* cassette flanked by the 5′- and 3′-termini of the *E. coli lacZ* sequence. The cassette was oriented in the same direction as the *lac* operon.

E. coli strain ATCC 47002 contains mutations that result in the inability to maintain plasmid pBRINT–Cm, which contains a ColE1 origin of replication. Cells were transformed with pKLN23-28 and selection with chloramphenicol resulted in strains that contained a *T7lac–glmS–Cm* cassette integrated at the *lacZ* deletion site in the chromosome. The cassette was then transferred by P1 phage transduction into strain 7101-17 (DE3), generating strain 2123-12 (*manXYZ8*, $\Delta nag::tet^R$ DE3, $\Delta lacZ$:: T7lac–glmS–Cm).

2.3. Generation of GlmS mutants resistant to product inhibition

Variants of *glmS* genes were generated by error-prone PCR using pKLN23-28 as template DNA. PCR conditions were essentially as described by Moore and Arnold (1996). PCR products were cloned into the backbone of pKLN23-28, resulting in a library of *T7lac* expression vectors containing various *glmS* mutants. Following transformation into the expression host 7101-17(DE3), clones with increased GlcN production were screened by a plate assay. This was done by stabbing

individual colonies into M9A plates which had been overlayed with soft agar containing cells of an *E. coli* GlcN auxotrophic strain as indicator. After overnight incubation, the halo size (growth area of indicator cells) reflected the level of GlcN production. Improved *glmS* genes were integrated into the *E. coli* chromosome as described above. Strain 2123-54 contains a *T7lac* expression cassette with a mutant *glmS* gene (*glmS*54*). PCR mutagenesis, mutant screening and characterization will be detailed elsewhere.

2.4. Integration of the T7lac–glmS*54 expression cassette at the galK site

By using a temperature shift protocol described by Hamilton et al. (1989), the *T7lac–glmS*54* expression cassette was integrated at the *galK* site in 7101-17(DE3), generating strain 7107-18. For that purpose, vector pSW07-9 was constructed with a kanamycin resistance marker (kan^R), a temperature-sensitive pSC101 replication origin and the *T7lac–glmS*54* expression cassette flanked by the *E. coli gal* sequence. A portion of the *gal* operon (*galTKM*, 3.3 kb, starting at 14 bp upstream of the ATG start codon of *galT* and ending at 68 bp downstream of the stop codon of *galM*) was amplified by PCR from *E. coli* W3110 genomic DNA and cloned into vector pPCR-Script^{TM}Amp SK(+). A 0.7-kb deletion was made in the *galK* sequence (between the restriction sites *Sfo*I and *Mlu*I) and restriction sites (*Mlu*I, *Sal*I, *Bgl*II, and *Msc*I) were added at the deletion site, generating plasmid pKLN07-1. A kanamycin resistance marker was isolated as a *Pst*I fragment from pUC4K (Amersham Pharmacia Biotech, Piscataway, NJ) and ligated into the *Not*I site of the plasmid pKLN07-1 after both were blunted with T7 DNA polymerase. The orientation of the kan^R marker in the resulting plasmid was not determined. The kan^R–*galTKM* sequence was isolated as a *Bam*HI/*Sac*II fragment, blunted with T4 DNA polymerase and ligated with a *Pvu*II/*Sma*I fragment containing the temperature-sensitive replication origin of pMAK705 (Hamilton et al., 1989), generating plasmid pSW07-4. Lastly, the expression cassette *T7lac–glmS*54*, including the *glmS* terminator, was isolated from pKLN23-54 as a *Not*I fragment, blunted with T4 DNA polymerase, and cloned into pSW07-4 at the *Msc*I site, generating plasmid pSW07-9. The *T7lac–glmS*54* cassette was placed in the same orientation as the *gal* operon. The plasmid was transformed into 7101-17(DE3). Sequence integration and subsequent removal of the vector sequence were carried out as described previously (Hamilton et al., 1989). The resulting strain 7107-18 was confirmed by Southern blot to contain the *T7lac–glmS*54* expression cassette integrated at the *galK* deletion site. The expression cassette was oriented in the same direction as the *gal* operon.

2.5. Cloning and expression of different GNA1 genes

Forward and reverse primers were designed to amplify by PCR the GlcN-6-P *N*-acetyltransferase genes from *Saccharomyces cerevisiae* (*ScGNA1*), *Candida albicans* (*CaGNA1*) and *Arabidopsis thaliana* (*AtGNA1*) according to the sequence information available at the web site of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The DNA templates used in PCR were from *S. cerevisiae* strain S288C, *C. albicans* strain ATCC10261 and *A. thaliana* BAC clone F14F8 (*Arabidopsis* Biological Resource Center DNA Stock Center, Columbus, OH). All the forward primers contained a *Bsa*I site and 21–31 nucleotides coding for the N-terminal peptide sequences, including the ATG start codon. All the reverse primers contained an *Xho*I site and 24–30 nucleotides coding for the C-terminal peptide sequences, starting from their stop codon.

ScGNA1 forward primer: 5′-GAT CGG TCT CGC ATG AGC TTA CCC GAT GGA TTT TAT ATA AGG C-3′.

ScGNA1 reverse primer: 5′-GAT CCT CGA GCT ATT TTC TAA TTT GCA TTT CCA CGC CTG C-3′.

CaGNA1 forward primer: (5′-GAT CGG TCT CGC ATG ATG TTA CCA CAA GGT TAT AC-3.

CaGNA1 reverse primer: 5′-GAT CCT CGA GCT AGA ATC TAC ATA CCA TTT CAA C-3′).

AtGNA1 forward primer: 5′-GAT GGT CTC GCA TGG CTG AGA CAT TCA AGA TC-3′.

AtGNA1 reverse primer: 5′-GAT CCT CGA GTT AAT CGA AGT ACT TAG ACA TTT GAA TC-3′.

PCR amplifications were conducted using the standard protocol to generate DNA fragments containing different *GNA1* coding sequences flanked by *Bsa*I and *Xho*I sites. The sequences were cloned between the *Nco*I and *Xho*I sites in the expression vector pET24d(+), creating plasmids with expression cassettes *T7lac–ScGNA1* (pSW07-62) , *T7lac–CaGNA1* or *T7lac–AtGNA1*.

2.6. Integration of multiple copies of T7lac–ScGNA1 expression cassette in the chromosome

Using cloning strategies and temperature selection protocols described above, the *T7lac–ScGNA1* expression cassette was integrated at the *manXYZ* locus, generating strain 7107-92. The expression cassette was isolated as an *Nae*I fragment from plasmid pSW07-62, starting at position 49 upstream of the T7 promoter and ending at position 164 downstream of the T7 terminator. At the insertion site, most of the *manXYZ* sequence was deleted by the removal of a 2647-bp *Hpa*I fragment.

To maximize *GNA1* expression, three additional copies of *T7lac–ScGNA1* cassette were integrated at the loci of *fucIK*, *treB* and *melRAB*, which encode for enzymes involved in the metabolism of L-fucose,

trehalose and melibiose, respectively. The target sites were chosen because these carbohydrates serve as alternative carbon sources. Although trehalose was also known to act as an osmoprotectant in *E. coli*, deletion and insertion at the *treB* locus did not noticeably affect growth and GlcNAc production in media containing glucose as the sole carbon source, as compared to strains with the wild type *tre* operon. The second copy of the cassette was integrated at the *fucIK* site, replacing a portion of the *fucI* and *fucK* genes (a 1239-bp *Hpa*I–*Bsr*GI fragment), generating strain 7107-607. A *Bgl*II–*Bsr*GI fragment of 149 bp in the *treB* coding sequence was deleted and replaced by the third copy of the *GNA1* cassette, generating strain 7107-608. A different strain with three copies of *GNA1* (strain 7107-609) was constructed by integrating the third copy of the cassette at the *melRAB* deletion site. The entire *melA* and the first 199 nucleotides of the *melB* sequences were deleted by the removal of a 1676-bp *Bgl*II-*Asi*SI fragment. Using 7107-608 as the parent strain, a fourth copy of the cassette was integrated at the *melRAB* site. All integrated copies of the *T7lac–ScGNA1* cassette were oriented in the same direction as the targeted operons.

2.7. Growth medium

The basic growth media used for GlcN or GlcNAc production were defined mineral salt media, M9A or M9B. M9A medium included the following macroelements (per liter): 14 g KH_2PO_4, 16 g K_2HPO_4, 1 g $Na_3Citrate \cdot 2H_2O$, 7.5 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4 \cdot 7H_2O$ and 0.015 g $CaCl_2 \cdot 2H_2O$. The pH was between 6.8 and 7.0 after sterilization. M9B medium contained (per liter) 6 g KH_2PO_4, 24 g K_2HPO_4, 1 g $Na_3Citrate \cdot 2H_2O$, 10 g $(NH_4)_2SO_4$, 0.6 g $MgSO_4 \cdot 7H_2O$ and 0.025 g $CaCl_2 \cdot 2H_2O$. The pH was between 7.0 and 7.2 after sterilization. Unless specified otherwise, the trace elements were supplied as follows (per liter, final concentration): 0.01 mg $CoCl_2 \cdot 6H_2O$, 0.01 mg H_3BO_3, 0.01 mg $CoSO_4 \cdot 5H_2O$, 0.5 mg $FeSO_4 \cdot 7H_2O$, 0.033 mg $MnSO_4 \cdot H_2O$, 0.38 mg $ZnSO_4 \cdot 7H_2O$ and 0.01 mg $NaMoO_4 \cdot 2H_2O$. The media were supplemented with glucose (20 g l^{-1}) as the carbon source, if not indicated otherwise.

2.8. Shake flask culture

A three-step protocol was developed to evaluate different production strains in shake flask experiments. First, cells freshly grown on LB plates were used to start cultures in 3 ml LB, which were grown at 37 °C for about 8 h. Secondly, a portion of the cultures (1.5 ml) was used to inoculate 50 ml of M9A medium in 250-ml shake flasks. Cultures were incubated at 37 °C and shaken at 225 rpm for about 16 h. Cell densities of the cultures were measured at 600 nm (OD_{600}). One unit of OD_{600} corresponded approximately to a cell density of 0.5 g l^{-1} dry weight. This culture step was included for cells to adapt to the minimal medium and for reproducible results of GlcN or GlcNAc production. Next, aliquots of the cultures were added to 50 ml of M9A or M9B containing IPTG (0.2 mM) in 250-ml shake flasks. The initial values of OD_{600} were set at 0.3. Cells were incubated at 37 °C and shaken at 225 rpm for 72 h. Samples of 1 ml were taken at 24, 48 and 72 h to measure cell density and levels of glucose, acetate, GlcN and/or GlcNAc. At 24 and 48 h, pH was adjusted to 7.0 by addition of a small amount of 10 M NaOH and the glucose level in the medium was adjusted to 20 g l^{-1} by addition of a concentrated glucose solution (65%, w/v).

2.9. GlcN production in 1-L fermentors

Fed-batch fermentation was run in 1-L fermentors. Fermentation medium contained the following components (per liter); 3.5 g KH_2PO_4, 4 g K_2HPO_4, 1 g $Na_3Citrate \cdot 2H_2O$, 5 g $(NH_4)_2SO_4$, 0.6 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot 2H_2O$ and 0.25 g antifoam agent Mazu DF204. These components were batch sterilized together. The pH was between 6.8 and 7.0 after sterilization. Unless specified otherwise, the same trace elements described above were added, but at 10-fold higher concentrations. A glucose solution (65%, w/v) was sterilized separately and added into the medium to the concentrations of 2–5 g l^{-1} before inoculation.

The medium volume before inoculation was 50% of the 1-L total fermentor capacity. Aeration was kept between 0.5 to 1 vvm. Dissolved oxygen was maintained at 20% saturation by controlling the rate of agitation. The growth temperature was maintained at 30 or 37 °C, and post-induction temperature at 25 or 30 °C (depending on the experiment). pH was controlled at 6.9 by automatic addition of 10 M NH_4OH, which also provided additional nitrogen for biomass and GlcN production.

Fermentors were inoculated with 2.5–5% culture (by volume) to an initial OD_{600} of about 0.5. Glucose was fed as a 65% solution (w/v) using a computerized program to control growth and minimize production of acetate. When an OD_{600} of 20–30 was reached, the culture was induced with lactose, either by a single point addition or slow feeding (up to 25 g l^{-1} lactose). Glucose was fed thereafter at a rate of 4–6.5 g l^{-1} h^{-1} to supply carbon for GlcN production. With glucose feed and base addition the final volume of the fermentation reached 75–80% of the total fermentor capacity. The fermentation was discontinued 72 h after inoculation.

2.10. GlcNAc production in 1-L fermentors

High GlcNAc titers were achieved by optimizing medium composition, lactose induction (timing and

dose) and glucose feeding. With respect to the protocol described above, a few changes were made. The optimized medium composition was as follows (per liter): 6.67 g KH_2PO_4, 3.55 g citric acid · H_2O, 0.025 g $CaCl_2 \cdot H_2O$, 2.5 g $MgSO_4 \cdot 7H_2O$ and 0.25 g antifoam agent Mazu DF204. These components were batch sterilized together before adding the following trace elements (per liter): 0.1 mg $CoCl_2 \cdot 6H_2O$, 0.1 mg $CuSO_4 \cdot 5H_2O$, 5 mg $FeSO_4 \cdot 7H_2O$, 0.33 mg $MnSO_4 \cdot H_2O$ and 3.8 mg $ZnSO_4 \cdot 7H_2O$. Glucose (65%, w/v) was sterilized separately and added to the medium (5 g per liter) before inoculation. The pH was near 3.0 after sterilization and was adjusted to 6.9 with 10 M NH_4OH before inoculation.

Fermentors were inoculated with 2.5–5% culture (by volume) to an initial OD_{600} of about 0.5% and 65% w/v glucose was fed on a program to limit growth to a rate of 0.4–0.45 h^{-1}. Throughout the fermentation, temperature was maintained at 37 °C. The pH was controlled at 6.9 by addition of 10 M NH_4OH. When an OD_{600} of 10 was reached, glucose feeding was set at a steady rate of 6.5 $g\,l^{-1}\,h^{-1}$ (based on initial volume) until the end of fermentation (for a total of 72 h). When an OD_{600} of 25–30 was reached, the culture was induced with a single addition of 5 $g\,l^{-1}$ lactose without suspension of the glucose feed.

2.11. Enzyme activity assays

GlcN-6-P synthase activity in crude enzyme extracts was measured by a coupled spectrophotometric assay using glutamate dehydrogenase as described previously (Badet et al., 1987). GlcN-6-P *N*-acetyltransferase was assayed by quantifying the amount of CoA released from acetyl-CoA following the method described by Mio et al. (1999).

2.12. GlcN and GlcNAc determination

GlcN in the culture medium was assayed by a modification of the colorimetric method of Elson and Morgan using 4-dimethylaminobenzaldehyde (Ehrlich's Reagent), essentially as described by Zalkin (1985). GlcNAc interfered with the colorimetric assay for GlcN. In samples which contained a significant amount of GlcNAc, GlcN was assayed by HPLC (Wayne et al., 2000) on a Phenomenex Prodigy ODS(3) C18 column (150 × 4.6 mm, 5 μm, Phenomenex, Torrance, CA) maintained at ambient temperature. The mobile phase was a MeOH:aqueous buffer (1:4 v/v) run at 0.7 $ml\,min^{-1}$. The aqueous buffer consisted of 10 mM sodium acetate and 10 mM sodium octanesulfonate, pH 5.1. GlcNAc was measured by HPLC using a Bio-Rad AMINEX HPX 87H Organic Analysis Column (300 × 7.8 mm) that was water-jacketed to maintain the column temperature at 40 °C. The mobile phase of 0.013 M nitric acid was run at 0.8 $ml\,min^{-1}$.

2.13. Determination of GlcN stability

GlcN stability was measured in uninoculated M9A medium supplemented with 40 $g\,l^{-1}$ glucose. GlcN was prepared as a 30% GlcN hydrochloride solution and was added to the desired final concentrations (50 ml total volume in 250-ml flasks). The pH of the medium was adjusted to pH 7.0, 6.5, 6.0 and 5.5. Flasks were incubated at 30 °C on a shaker and agitated at about 225 rpm. Samples of 1 ml were taken at intervals for analysis. After each sampling, pH in the flasks was measured by submerging a pH probe in the liquid and adjusted to the initial values by adding droplets of a 5-N NaOH solution. GlcN levels were determined by the colorimetric method.

2.14. Effects of GlcN on E. coli strain 7107-18

Cells were inoculated into M9A medium supplemented with glucose (40 $g\,l^{-1}$) and GlcN at different concentrations: 0, 10, 20 and 40 $g\,l^{-1}$. Cell density (OD_{600}) and numbers of colony-forming units were determined in samples taken at intervals. To test the involvement of GlcN degradation products in growth inhibition, growth medium containing different levels of GlcN were prepared and incubated at 30 °C for 4, 8 and 16 h to allow degradation products to form. Cells were inoculated into the media and cell growth in these cultures was compared to cultures that were inoculated immediately (within 30 min) after the media had been prepared.

2.15. GlcNAc hydrolysis

The conversion of GlcNAc to GlcN was examined using a 1% solution of GlcNAc incubated at various temperatures (35, 60 and 100 °C) in the presence of HCl (0–1.0 M). Samples were taken over time and analyzed by HPLC for GlcNAc and GlcN.

3. Results

3.1. Gene inactivation and glmS over-expression

An *E. coli* K-12 strain (ATCC 25947) was used as the host for metabolic engineering. The genotypes of the host strain and key recombinant strains are listed in Table 1. The host strain was modified to minimize the transport and catabolism of GlcN and GlcNAc. This was achieved by introducing a mutation in the *manXYZ* operon and deleting the *nag* regulon (Fig. 1). A λ-DE3 element containing a T7 RNA polymerase gene under

Table 1
E. coli strains

Strain no.	Genotype	Remark	Reference
ATCC 25947[1)]	F⁻ λ⁻ *rph-1* IN(*rrnD-rrnE*)*1 Thy*, a derivative of *E. coli* K-12 strain W3110	Parent strain for pathway engineering	ATCC
IBPC566	*thi*-1 *arg*G6 *arg*E3 *his*-4 *mtl*-1 *xyl*-5 *rpsL tsx*-29? Δ*lac*X74 *man*XYZ8 *zdj*-225::Tn10		Plumbridge (1990)
IBPC590	*thi-1 argG6 argE3 his-4 mtl-1 xyl-5 rpsL tsx-29? ΔlacX74 Δnag::tet*^{R}		Plumbridge (1992)
ATCC 47002	*F⁻ recB21 recC22sbcB15 leu-6 ara-14 his-4 λ⁻*		ATCC
7101-17 (DE3)	Δ*nag*::*tet*^{R} *manXYZ8* DE3	Derived from ATCC 25947	This work
2123-12	Δ*nag*::*tet*^{R} *manXYZ8* DE3 Δ*lacZ*:: T7lac–glmS–Cam^R	Over-expression of the *E. coli glmS* gene integrated at the *lacZ* locus, derived from 7101-17(DE3)	This work
2123-54	Δ*nag*::*tet*^{R} *manXYZ8* DE3 Δ*lacZ*:: T7lac–glmS*54–Cam^R	Over-expression of a mutant *E. coli glmS* gene integrated at the *lacZ* locus, derived from 7101-17(DE3)	This work
7107-18[2)]	Δ*nag*::*tet*^{R} *manXYZ8* DE3 Δ*galK*::T7lac–glmS*54	Over-expression of a mutant *E. coli glmS* gene integrated at the *galK* locus, derived from 7101-17(DE3)	This work
7107-88	7107-18, pET24d(+) Kan^R	Hosting an empty vector	This work
7107-87	7107-18, pET24d(+)/T7lac–ScGNA1 Kan^R	Hosting an expression vector containing the *Saccharomyces cerevisiae GNA1* gene	This work
7107-93	7107-18, pET24d(+)/T7lac–AtGNA1 Kan^R	Hosting an expression vector containing the *Arabidopsis thaliana GNA1* gene	This work
7107-117	7107-18, pET24d(+)/T7lac–CaGNA1 Kan^R	Hosting an expression vector containing the *Candida albicans GNA1* gene	This work
7107-92	7107-18, Δ*manXYZ*::T7lac–ScGNA1	One copy of integrated *ScGNA1* expression cassette	This work
7107-607	7107-18, Δ*manXYZ*::T7lac–ScGNA1 Δ*fucIK*::T7lac–ScGNA1	Two copies of integrated *ScGNA1* expression cassette	This work
7107-608	7107-18, Δ*manXYZ*::T7lac–ScGNA1 Δ*fucIK*::T7lac–ScGNA1 Δ*treB*::T7lac–ScGNA1	Three copies of integrated *ScGNA1* expression cassette	This work
7107-609	7107-18, Δ*manXYZ*::T7lac–ScGNA1 Δ*fucIK*::T7lac–ScGNA1 Δ*melAB*::T7lac–ScGNA1	Three copies of integrated *ScGNA1* expression cassette	This work
7107-612	7107-18, Δ*manXYZ*::T7lac–ScGNA1 Δ*fucIK*::T7lac–ScGNA1 Δ*treB*::T7lac–ScGNA1 Δ*melAB*::T7lac–ScGNA1	Four copies of integrated *ScGNA1* expression cassette	This work

Note: (1) Except IBPC566, IBPC590 and ATCC47002, all other strains listed in the table were derived from the same parent strain ATCC 25947. (2) Strains listed below this lane were all derived from strain 7107-18. For clarity, the genotypes of these strains are shown as 7107-18 plus additional modifications.

control of the *lacUV5* promoter was integrated in the chromosome by lysogenization so that the T7-based expression system could be used to over-express biosynthesis genes (Studier and Moffatt, 1986), generating strain 7101-17(DE3). The *E. coli glmS* coding sequence was cloned and placed under *T7lac* promoter control, forming a *T7lac–glmS* expression cassette in plasmid pET24d(+). The expression cassette was then integrated into the chromosome of the strain 7101-17(DE3) at the *lacZ* locus, generating strain 2123-12. Different *E. coli* strains were grown at 37 °C in the defined mineral salt medium M9A containing 1 mM isopropylthio-β-D-galactoside (IPTG). In strain 2123-12 GlmS protein was expressed as a predominant protein as observed on SDS-PAGE (data not shown). GlmS activity in the parent strain 7101-17(DE3) was very low (around 10 nmol min^{-1} mg^{-1} protein) while it reached about 500 nmol min^{-1} mg^{-1} protein in 2123-12. The amount of GlcN detected in the growth medium was typically 4 mg l^{-1} with the wild type *E. coli* strain at 26 h. The level reached 75 mg l^{-1} with strain 2123-12.

3.2. Generation and over-expression of GlcN synthases resistant to product inhibition

Mutant variants of the *E. coli glmS* gene were generated by error-prone PCR. Improved enzymes were identified by screening for higher GlcN producers using a cross-feeding plate assay. From a population of about 9000 clones, 84 clones were confirmed to produce GlcN

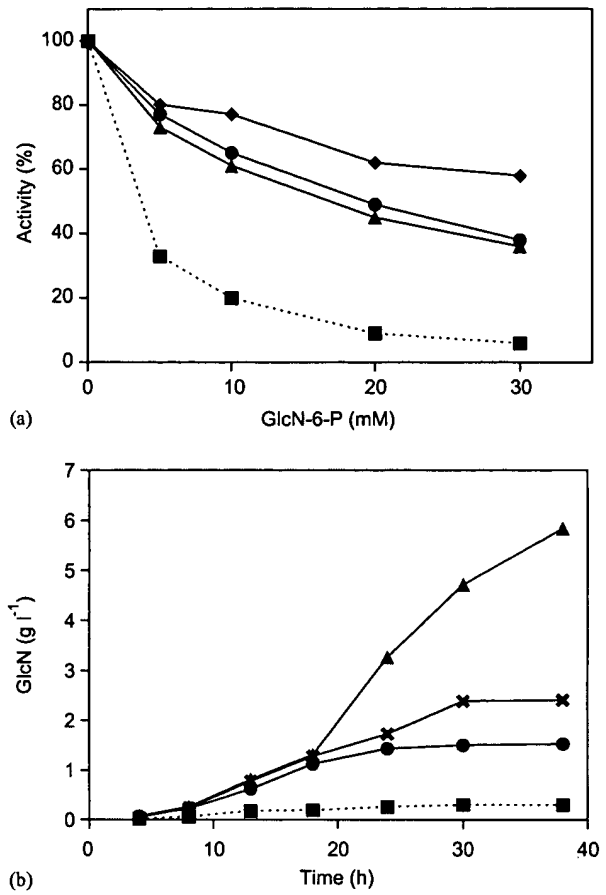

Fig. 2. GlmS mutants resistant to product inhibition. Strain 2123-12 (—■—, dotted line) is a strain containing an integrated *T7lac–glmS* expression cassette (with the wild type *E. coli glmS* gene). Other *E. coli* strains contain an integrated expression cassette with an *E. coli glmS* mutant gene generated through error-prone PCR: 2123-49 (—●—), 2123-51 (—✱—), 2123-54 (—▲—) and 2123-124 (—◆—). (a) GlmS activity determined in the presence of various concentrations of GlcN-6-P. (b) GlcN production in shake flask cultures induced with 1 mM IPTG at time of inoculation.

at levels significantly higher than strain 2123-12. Expression constructs with mutant *glmS* genes (*glmS**) were integrated into the chromosome at the *lacZ* site. Several mutant GlmS enzymes showed significantly reduced sensitivity to product inhibition (Fig. 2a). One of the best mutant strains, 2123-54, over-expressing the mutant *glmS* gene (*glmS*54*), produced 6 g l^{-1} GlcN (Fig. 2b). This was 20-fold higher than strain 2123-12 that over-expressed the wild-type *E. coli glmS* gene.

3.3. Lactose induction of GlcN production

In strain 2123-54 the *T7lac–glmS*54* expression cassette was integrated into the chromosome at the *lacZ* locus, resulting in inactivation of the *lac* operon. Genes under T7 promoter control require induction of the T7 RNA polymerase that is under *lacUV5* promoter control in the DE3 element. GlcN production in this strain was inducible by IPTG, which is too expensive to be used in large-scale fermentation. A more cost-effective alternative is to use lactose as an inducer. This requires a functional LacZ enzyme (β-galactosidase) to convert lactose to allolactose, the true inducer. Therefore, strain 7107-18 was developed by integrating the *T7lac–glmS*54* cassette at an alternate integration site. The *galK* locus was chosen as the integration site so that galactose could also be used as inducer (Mattanovich et al., 1998).

Glucose is often the preferred low-cost carbon source in industrial scale fermentations. Lactose induction with the T7 expression system is subject to repression by glucose. This problem can usually be minimized by appropriate process conditions (Neubauer et al., 1992; Hoffman et al., 1995). Lactose induction of GlcN production was studied in 1-L fermentors. Induction after an initial growth phase was found to be most effective for GlcN production. Cells were grown with glucose to an OD_{600} of about 20. The feed was switched from glucose to lactose for about 10 h, after which glucose feeding was resumed. Using this protocol, GlcN levels reached 17 g l^{-1} by 72 h (Fig. 3a).

3.4. Degradation of GlcN and effects of GlcN on cell growth

Further GlcN titer improvement was impaired by a number of factors. These included GlcN instability as well as inhibition of cell growth by GlcN and its degradation products. GlcN degradation was found to be strongly pH-dependent, occurring faster at higher pH (Fig. 4a). When GlcN (60 g l^{-1}) was incubated at 30 °C in an uninoculated M9A medium adjusted to pH 7.0, as much as 68% of GlcN was degraded within 52 h. GlcN was stable when pH was below 4.8. An amber color developed as GlcN degraded.

Inhibitory effects of GlcN and its degradation product(s) on *E. coli* 7107-18 cells were demonstrated by growing cells in a medium containing different levels of GlcN (Fig. 4b). Growth was slightly inhibited by GlcN at 10 and 20 g l^{-1}. GlcN at 40 g l^{-1} completely prevented cell growth. After 16 h of incubation with GlcN, numbers of colony-forming units declined, indicating cell death. After 52 h, the viable cell count leveled off at about one-fifth of the original number (data not shown). When cells were inoculated in media which had been pre-incubated with GlcN at 35 g l^{-1} for 8 h, growth inhibition and cell death were more severe, suggesting that GlcN degradation product(s) are even more inhibitory to the host cell than GlcN.

To minimize product loss due to degradation, GlcN production at low pH was evaluated. *E. coli* cells were grown at normal optimal pH (6.5–7.0), induced with lactose and then switched to pHs below 5.0. This

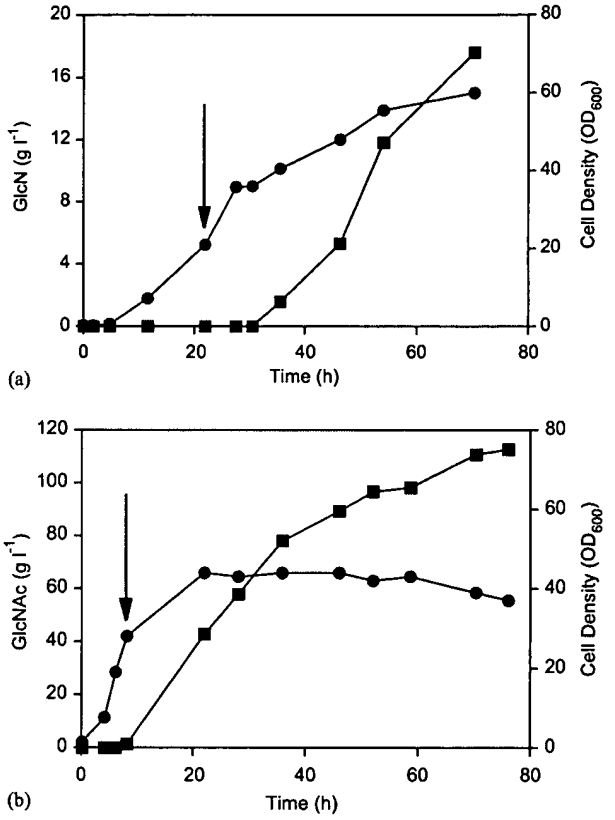

Fig. 3. Production of GlcN and GlcNAc in one-liter fermentors. Fermentations were run in a fed-batch mode. Cell density (—●—, OD_{600}), GlcN and GlcNAc titers (—■—) were monitored. (a) GlcN production using *E. coli* strain 7107-18 containing an integrated *T7lac–glmS*54* expression cassette. Cells were grown to an OD_{600} of 22 and induced by lactose ($30\,g\,l^{-1}$) added during a 10-h period as shown by the arrow. (b) GlcNAc production using *E. coli* strain 7107-607 containing an integrated *T7lac–glmS*54* expression cassette and two integrated copies of a *T7lac–ScGNA1* expression cassette. Cells were grown to an OD_{600} of 27 and induced with lactose ($5\,g\,l^{-1}$) added as a single pulse as shown by the arrow.

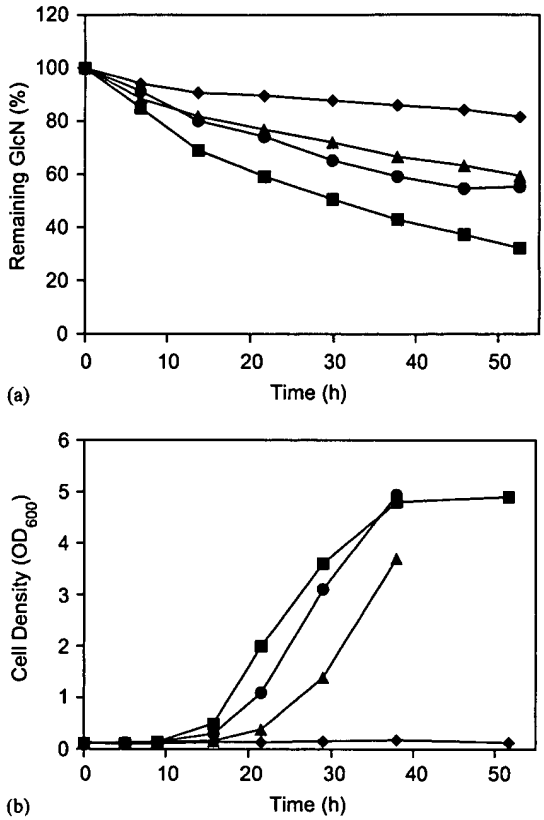

Fig. 4. GlcN degradation and inhibition of cell growth by high levels of GlcN. (a) GlcN degradation in an uninoculated growth medium at different pH: 5.5 (—◆—), 6.0 (—▲—), 6.5 (—●—) and 7.0 (—■—). The initial concentration of GlcN was $60\,g\,l^{-1}$. (b) Cell densities in cultures of *E. coli* strain 7107-18 incubated with various levels of GlcN in the medium: control (no GlcN, —■—), 10 (—●—), 20 (—▲—) and $40\,g\,l^{-1}$ (—◆—).

improved GlcN production by about 20% (data not shown).

3.5. Over-expression of GlcN-6-P N-acetyltransferase and GlcNAc production

To overcome the problems of GlcN degradation, and inhibitory effects of GlcN and its degradation products, GlcNAc was chosen as an alternative fermentation product. GlcNAc is stable in solution at neutral pH and the presence of $60\,g\,l^{-1}$ GlcNAc in the medium did not inhibit the growth of *E. coli* strain 7107-18 (data not shown). Moreover, GlcNAc was readily deacetylated to GlcN under relatively mild acidic conditions. More than 98% of GlcNAc ($10\,g\,l^{-1}$) was converted to GlcN by treatment with 0.1 M HCl at 100 °C for 3 h.

The GlcN pathway in strain 7107-18 was altered for the production of GlcNAc by expressing a heterologous *GNA1* gene that encodes for GlcN-6-P *N*-acetyltransferase (Fig. 1). Three different *GNA1* genes were expressed in *E. coli* for GlcNAc production. *GNA1* genes from *Saccharomyces cerevisiae* (*ScGNA1*), *Candida albicans* (*CaGNA1*) and *Arabidopsis thaliana* (*AtGNA1*) were cloned into the expression vector pET24d(+). Recombinant plasmids were transformed into the GlcN production *E. coli* strain 7107-18. *GNA1* transformants were grown in the M9B medium in shake flasks and induced with 0.1 mM IPTG. SDS-PAGE analysis confirmed expression of GNA1 proteins of expected sizes. All strains exhibited comparable levels of GlmS activity (Table 2). As expected, the control strain bearing the empty vector did not show a significant level of GNA1 activity. Expression of the yeast and higher plant *GNA1* genes led to high acetyltransferase activity in *E. coli*.

Under IPTG-induced conditions, *GNA1* transformants secreted high levels of GlcNAc in to the culture medium (Table 2). Little or no GlcN (less than $0.5\,g\,l^{-1}$) could be detected in the medium. This suggests that

Table 2
GNA1 expression and its impact on cell density, acetate and GlcNAc production

Strain	Construct[a]	Activity[b]		OD_{600}	Acetate ($g l^{-1}$)	GlcNAc ($g l^{-1}$)
		GlmS	GNA1			
7107-88	Vector	0.53	0.1	3.75	4.4	ND[c]
7107-87	*S. cerevisiae GNA1*	0.48	19.0	7.80	ND	11.7
7107-117	*C. albicans GNA1*	0.36	22.3	10.00	0.5	5.1
7107-93	*A. thaliana GNA1*	0.32	6.0	8.70	ND	8.0

[a]Different *GNA1* coding sequences were cloned into pET24d(+), forming *T7lac–GNA1* expression constructs. *E. coli* strains with these plasmids were grown for 23 h in M9B medium supplemented with 40 $g l^{-1}$ glucose, 10 $g l^{-1}$ ribose and 5 $g l^{-1}$ yeast extract. Cultures were induced with 0.2 mM IPTG at the time of inoculation.
[b]Enzyme activities are expressed in $\mu mol\, min^{-1}\, mg^{-1}$ protein.
[c]ND: not detectable.

high-level expression of the acetyltransferase prevented a buildup of the intermediate GlcN-6-P. Indeed, preliminary data showed that the intracellular levels of GlcN-6-P were about 15-fold lower in the GlcNAc production strain 7107-87 than in the GlcN production strain 7107-18. Among the three *GNA1* genes tested, the *S. cerevisiae* gene outperformed the others and was selected for further strain development.

3.6. Physiology of GlcNAc production

The strain hosting the plasmid with a *T7lac–ScGNA1* cassette (7107-87) was studied under different growth conditions. In the simple mineral salt medium M9B with glucose as carbon source, the strain grew to an OD_{600} of 5.4 after 24 h under non-induced conditions. However, when IPTG was added at the time of inoculation, the OD_{600} at 24 h was about 30% lower. Supplementation with yeast extract improved cell growth and GlcNAc production (Table 3). Pentose sugars (ribose in particular), or gluconate also had positive effects. The effects of yeast extract were additive to those of ribose and gluconate. Addition of both ribose and yeast extract resulted in a GlcNAc level of 22.4 $g l^{-1}$ at 48 h in shake flasks. On the other hand, GlcN production in cultures of 7107-18 was not affected by the addition of yeast extract, pentose and gluconate (data not shown).

Strains that produced high levels of GlcNAc accumulated little or no acetate in shake flask cultures under conditions where the control strain accumulated acetate at several grams per liter (Table 2). It is important to note that the GlcNAc-producing strain grew to much higher cell densities than the control strain (Table 2) under the same growth conditions.

3.7. Optimization of GlcNAc production process

A strain bearing the *ScGNA1* plasmid (7107-87) was grown in fermentors and induced with IPTG at the time of inoculation (early induction) or after 24 h of incubation (late induction). As shown in Fig. 5, cells grew very poorly and little GlcNAc was produced ($< 5 g l^{-1}$) when the culture was induced early. Addition of yeast extract slightly improved growth but did not increase GlcNAc production in early-induced cultures. On the other hand, the late induction allowed good growth and high GlcNAc production (50 $g l^{-1}$ at 70 h). In late-induced cultures, yeast extract and ribose increased GlcNAc production by improving cell growth

Table 3
Effects of medium supplementations on growth and GlcNAc production in shake flask cultures

Medium supplementation	OD_{600}	GlcNAc ($g l^{-1}$)
Experiment I: Lactose induction		
Lactose (40 $g l^{-1}$)	3.9	2.3
Lactose (40 $g l^{-1}$), yeast extract (5 $g l^{-1}$)	6.9	6.0
Lactose (30 $g l^{-1}$), glucose (10 $g l^{-1}$)	5.3	1.3
Lactose (30 $g l^{-1}$), glucose (10 $g l^{-1}$), yeast extract (5 $g l^{-1}$)	12.0	9.0
Experiment II: IPTG induction		
Glucose (30 $g l^{-1}$)	3.8	3.0
Glucose (20 $g l^{-1}$), ribose (10 $g l^{-1}$)	5.7	6.0
Glucose (30 $g l^{-1}$), yeast extract (5 $g l^{-1}$)	8.7	9.9
Glucose (20 $g l^{-1}$), ribose (10 $g l^{-1}$), yeast extract (5 $g l^{-1}$)	12.0	18.7
Experiment III: IPTG induction in a medium containing glucose (30 $g l^{-1}$) and yeast extract (5 $g l^{-1}$)		
Control	10.0	11.1
Ribose (10 $g l^{-1}$)	13.3	22.4
Xylose (10 $g l^{-1}$)	11.0	12.7
Arabinose (10 $g l^{-1}$)	11.5	15.2
Potassium gluconate (10 $g l^{-1}$)	10.0	18.7

E. coli strain 7107-87 hosting a free-replicating plasmid containing a *T7lac–ScGNA1* expression cassette were grown for 48 h in the defined mineral salt medium M9B with indicated supplementations. Cultures were induced, at the time of inoculation, with IPTG (0.2 mM) or lactose as indicated.

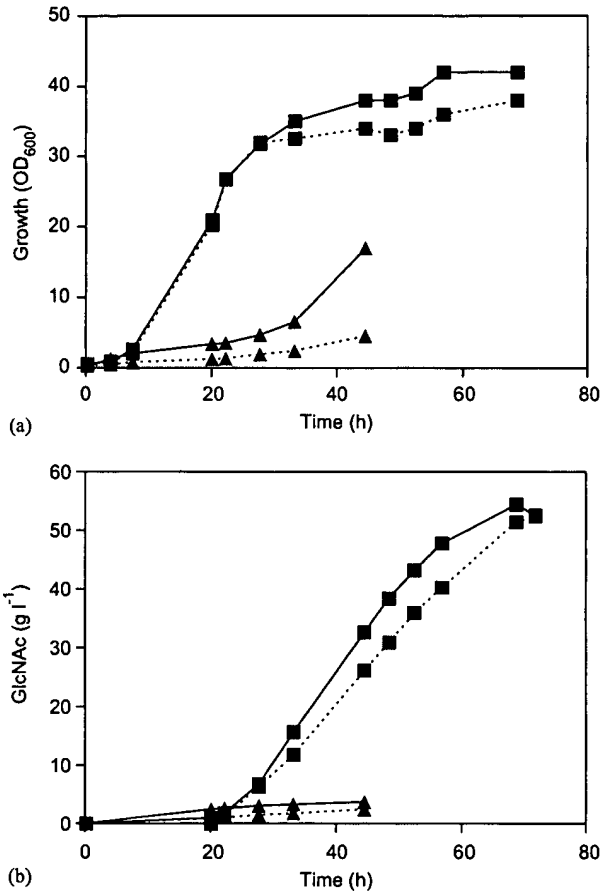

Fig. 5. Effects of the timing of IPTG induction on growth and GlcNAc production. *E. coli* strain 7107-87 (containing a *T7lac–glmS*54* expression cassette integrated in the chromosome and a *T7lac–ScGNA1* expression cassette carried on a free-replicating plasmid) was grown in one-liter fermentors. Cultures were induced with 0.4 mM IPTG at the time of inoculation (triangles) or 24 h after inoculation (squares). The medium was supplemented without (dotted lines) or with $4\,g\,l^{-1}$ yeast extract (solid lines). (a) Growth monitored by OD_{600}. (b) GlcNAc production.

without affecting the rate of specific formation (GlcNAc $g\,h^{-1}\,g^{-1}$ dry cell weight). Further experiments showed that these supplements were not required during fermentation when cell cultures were grown initially to a high cell density before induction.

Using the strain containing the *T7lac–ScGNA1* expression plasmid, lactose induction of GlcNAc production was relatively ineffective compared to IPTG induction under some experimental conditions. Four copies of the expression cassette *T7lac–ScGNA1* were integrated into the chromosome, each at a different target site. With the integrated cassette, lactose was as efficient as IPTG in inducing GlcNAc production. Strains with one, two and three copies of integrated cassette showed 25%, 50% and 70% GNA1 activity, respectively, as compared to a plasmid-bearing strain. GNA1 activity was not rate limiting, as levels of GlcNAc production in shake flasks and fermentors with single or multiple copies of the gene were comparable. During fermentation, GNA1 activity was elevated soon after induction and remained high throughout the 72-h run. GlmS activity was more variable, tending to decline towards the end of fermentation. Typically, high levels of GlmS activity towards the end were associated with high titers of GlcNAc.

To maximize GlcNAc production, fermentation experiments were conducted to evaluate inoculation cell density, lactose induction timing, lactose dose, glucose feeding rate, and medium composition, especially levels of phosphate, calcium and trace elements. With the improved fermentation conditions, GlcNAc production levels using strains containing two integrated copies of the *GNA1* cassette reached over $110\,g\,l^{-1}$ at 72 h (Fig. 3b). Levels of GlcN were below $1\,g\,l^{-1}$ throughout the fermentation. The process was successfully scaled up to 14- and 250-L fermentors with comparable titers. Product yield on glucose is one of the most important issues in developing a cost effective fermentation process. The maximal theoretical yield of GlcNAc on glucose is 67% by mole and 82% by weight. Due to the need for biomass production, the highest achievable yield on glucose by weight is about 70%. The weight yield on glucose in the GlcNAc fermentation described above was around 45%. With high product titers already achieved, current research is focused on maximizing the yield on glucose yield through strain and process optimization, and scaling up the fermentation process to industrial manufacturing scale.

4. Discussion

Metabolic engineering is a powerful tool that can be used to increase the capacity and efficiency of current production hosts or to create new hosts to produce novel chemicals by using advanced methods of metabolic flux analysis and molecular genetics such as gene inactivation, over-expression, deregulation, recombination and directed evolution. Metabolic engineering was successfully used in the present work to develop microbial strains for fermentation production of GlcN and GlcNAc.

E. coli was modified to accumulate GlcN by inactivating genes involved in GlcN transport and catabolism, and by over-expressing the biosynthesis gene (*glmS*). As a result, the GlcN level was increased from about 4 to $75\,mg\,l^{-1}$. GlmS is known to be sensitive to product inhibition (White, 1968; Broschat et al., 2002). GlcN-6-P strongly inhibits GlmS activity while GlcN only slightly inhibits the enzyme. Directed evolution has been increasingly used to improve enzyme characteristics (Moore and Arnold, 1996; Castle et al.,

2004; Lutz and Patrick, 2004). Mutant variants of the *E. coli glmS* gene were generated by error-prone PCR and clones producing GlcN at higher levels were screened by a cross-feeding plate assay. Sensitivity to production inhibition was significantly reduced in GlcN over-producing mutants. Using one of the best mutants, the level of GlcN produced in shake flask culture was 15 fold higher than the strain over-expressing the wild-type enzyme.

In general, amino sugars containing a free amino group are unstable in aqueous solution at neutral pH. GlcN has been reported to undergo spontaneous rearrangement and dimerization, forming fructosazine, D-arabinose, 5-(hydroxymethyl)-2-furaldehyde, 2,5-bis (tetrahydroxybutyl) pyrazine and other undetermined products (Kashige et al., 1995; Zhang et al., 2003). Rapid degradation of GlcN was a barrier to higher GlcN titers. In addition, GlcN was shown to inhibit cells of *E. coli* 7107-18. This is at least partially due to the deletion of the *nagB* gene. Despite the mutation in the mannose transporter operon *manXYZ*, GlcN can still be taken up via the glucose transporter, potentially leading to toxic levels of intracellular GlcN-6-P. High intracellular levels of phosphorylated sugars and amino sugars are toxic, inhibiting enzymes and causing degradation of specific mRNA species, cell lysis or stasis (White, 1968; Bernheim and Dobrogosz, 1970; Kadner et al., 1992; Park, 2001; Morita et al., 2003). Moreover, inhibitory effects of GlcN on *E. coli* 7107-18 were more pronounced in cultures where GlcN degradation products were formed before inoculation, suggesting that GlcN degradation product(s) are even more detrimental to the host cell. It was shown that GlcN degradation products, such as dehydrofructozane, cleaved plasmid DNA in vitro (Kashige et al., 1995). The mechanism(s) by which GlcN degradation products affect *E. coli* cells are not known.

To minimize GlcN degradation, alternative fermentation strategies were explored. After a growth period at a pH that was optimal for cell growth pH (6.5–7.0), pH in the fermentor was shifted to below 5.0 for GlcN production. Preliminary experiments showed only marginal improvement in GlcN titer. It would be challenging to develop an efficient *E. coli* fermentation process operating at low pH since *E. coli* generally has very low metabolic activity below pH 5.0. As an alternative, the GlcN synthesis pathway is being manipulated in yeast and lactic bacteria, which grow well at acidic pH (data not shown).

For the fermentation process using *E. coli*, another alternative was to produce a stable derivative of GlcN. GlcNAc was found to be an ideal alternative product since it is stable in solution at neutral pH and does not inhibit the growth of *E. coli* strain 7107-18. Moreover, GlcNAc was readily hydrolyzed to GlcN under relatively mild acidic conditions. Therefore, the GlcN pathway in strain 7107-18 was altered for the production of GlcNAc. Unlike bacteria, in yeast and other eukaryotic organisms, GlcN-6-P *N*-acetyltransferase (encoded by the *GNA1* gene) catalyzes the formation of GlcNAc-6-P (Mio et al., 1999). Yeast and plant *GNA1* genes were expressed in *E. coli*. This led to production of GlcNAc at significant levels.

Cell growth was inhibited when *GNA1* expression was induced at the time of inoculation, which appeared to be caused by amino sugar phosphate toxicity discussed above. It has been speculated that the accumulation of amino sugar phosphates affected growth by inhibiting glucose-6-P dehydrogenase (Bernheim and Dobrogosz, 1970). This would ultimately result in a shortage of pentose phosphate, metabolites important for the cell multiplication, especially in nucleotide synthesis. After IPTG or lactose induction the intracellular levels of GlcNAc-6-P could reach a level that is inhibitory to enzyme(s) involved in pentose phosphate pathway. Indeed, supplementation with pentose sugars and gluconate improved cell growth and GlcNAc production. Yeast extract also improved growth and GlcNAc production. On the contrary, addition of pentose and yeast extract did not affect GlcN production in *E. coli* 7107-18. The inhibitory effects of GlcNAc production on host cells were effectively minimized by inducing GlcNAc production after the culture had reached a high cell density.

By optimizing fermentation medium and other process conditions, GlcNAc level in the medium reached $110\,g\,l^{-1}$. Interestingly, levels of GlcN were always below $1\,g\,l^{-1}$, indicating very efficient conversion of GlcN-6-P to GlcNAc-6-P. The latter was also efficiently dephosphorylated and secreted into the medium. It is generally accepted that phosporylated intermediates are dephosphorylated during export from *E. coli*. The *E. coli* periplasmic space contains several phosphatases, including alkaline phosphatase and sugar phosphatases that could dephosphorylate GlcN-6-P and GlcNAc-6-P (Wanner, 1996).

Accumulation of acetate is a common obstacle to achieving high levels of recombinant protein and other fermentation products in *E. coli* (Luli and Strohl, 1990; Chen and Bailey, 1993). With excess glucose in the medium, *E. coli* cells tend to synthesize high levels of acetate and other organic acids, usually resulting in growth inhibition. Acetate production was a problem confronted in the development of the GlcN production process. GlcNAc synthesis consumes acetyl-CoA, the precursor for acetate formation. Although use of acetyl-CoA is potentially a metabolic burden on the cell, the redirection of acetyl-CoA to GlcNAc production apparently represents a significant benefit by helping to avoid acetate accumulation.

Overall, there appear to be multiple factors contributing to high GlcNAc production. GlcNAc in the medium

is inert to the host cell due to inactivation of specific transporters. *GNA1* over-expression had a dramatic impact on the pathway of amino sugar synthesis in *E. coli*. Phosphorylated amino sugar intermediates cause inhibition of growth but this was minimized by establishing a two-phase fermentation protocol. The acetylation step catalyzed by GNA1 acted as a strong driving force to decrease intracellular GlcN-6-P levels and increase the metabolic flux to GlcNAc. The synthesis of GlcNAc also consumed acetyl-CoA, and as a consequence, minimized acetate formation.

Metabolic engineering for GlcN and GlcNAc production started with a straightforward strategy: inactivate genes involved in GlcN transport and catabolism, and over-express the biosynthesis gene (*glmS*). This strategy led to a 15-fold increase in GlcN production, but the titer remained at the milligram level. Product inhibition of GlmS by GlcN-6-P was identified as a critical factor in regulating the pathway. Directed evolution of the GlmS enzyme and over-expression of a product-resistant GlmS proved to be very effective in increasing GlcN production, and the titer reached multi-gram levels. However, rapid GlcN degradation and inhibitory effects of GlcN and its degradation products on the host cell prevented further titer improvement. Extending the pathway to GlcNAc, an amino sugar that is stable and inert to the *E. coli* host, by over-expressing a heterologous GlcN-6-P *N*-acetyltransferase gene (*GNA1*) represented a significant conceptual breakthrough. Finally, development and optimization of a two-phase (growth phase followed by production phase) fed batch process minimized inhibitory effects of phosphorylated amino sugars on the host cell, and resulted in the development of a high-performance fermentation process for the production of GlcNAc.

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